REMARKS

Rejection of Claims 25 and 44-46 Under 35 U.S.C. § 103:

The Examiner has maintained the rejection of Claims 25 and 44-46 under 35 U.S.C. §103, contending that these claims are unpatentable over Braxton in view of the teachings of Boissel et al., Weich et al. and Lin et al. The Examiner presents two arguments in support of this rejection, which are discussed below.

Applicant respectfully traverses the Examiner's rejection of Claims 25 and 44-46 under 35 U.S.C. § 103. In determining obviousness, one must focus on Applicant's invention as a whole. *Symbol Technologies Inc. v. Opticon Inc.*, 19 U.S.P.Q.2d 1241, 1246 (Fed. Cir. 1991). The primary inquiry is: "whether the prior art would have suggested to one of ordinary skill in the art that this process should be carried out and would have had a reasonable likelihood of success . . . . Both the suggestion and the expectation of success must be found in the prior art, not in the applicant's disclosure." *In re Dow Chemical*, 5 U.S.P.Q.2d 1529, 1531 (Fed. Cir. 1988). A conclusion of obviousness requires that the reference(s) relied upon be enabling in that it put the public in possession of the claimed invention. *In re Hoeksema*, 399 F.2d 269, 274, 158 USPQ 596, 601 (CCPA 1968). With these standards in mind, the Examiner's rejections are discussed below.

Reasons why the Examiner is requested to consider these arguments and the attached publication after final rejection are presented below. The Examiner's review of these reasons is respectfully requested.

First, in response to Applicant's argument that there is insufficient certainty in the art regarding the requirement of the C-terminal region of EPO for its activity, the Examiner responds by asserting that while the references provided by Applicant in support of this position would indicate that one of skill in the art would not have a reasonable expectation of success at making deletion mutants at the C-terminus, these teachings are not[sic] sufficient to indicate an absence of an expectation of success in making the claimed invention. The Examiner contends that because the present claims read on the addition of a cysteine to the end of EPO, rather than a substitution, the modification is more akin to making a fusion of EPO at its C-terminus. The Examiner contends that three instances of such EPO fusions were found in the art, two of which resulted in an active fusion protein. The Examiner points to Weich and Mele in support of this

argument. With respect to an inactive fusion in Nielson et al., the Examiner argues that while fusion may have been the cause of the inactivity, so may have been the extreme denaturing conditions used to extract the protein, and since Weich and Mele are later publications, one of skill in the art would assume the problem in Nielson et al. was the purification process.

The Examiner's full consideration of the following argument in response to the current rejection is respectfully requested. It is noted that even though this is a final rejection, the Examiner's position has changed from the initial position that the art teaches that the "C-terminal four residues of the protein are not required or involved in protein function" (see November 28, 2006 Office Action, page 3), to an apparent *acknowledgement* that the modification of the C-terminal residues may impact function, along with a shift of argument to the position that only *additions* to the C-terminus of EPO, as in a fusion protein, are relevant to the rejection. In addition, the Examiner has added the reference of Mele (U.S. Patent No. 5,916,773) to the supporting evidence for the rejection, which Applicant has not previously had an opportunity to address. Therefore, Applicant requires an opportunity to reply to this newly stated position and additional reference of the Examiner.

The Examiner contends that given that two out of three EPO fusion proteins described in the art retained activity, this is sufficient evidence that the C-terminus of EPO can be modified by addition of a cysteine residue and still retain activity. Applicant submits that the Examiner has not established through citation of any of these references that addition of *a cysteine residue* to the C-terminus of EPO would predictably result in a biologically active protein. With regard to Weich and Mele, each of these references constructed a fusion between EPO and a large growth factor protein using a linker formed from glycine, serine and/or alanine, all commonly used in linkers because they are smaller, relatively non-reactive amino acids. For example, the newly cited reference of Mele describes an EPO-L-GM-CSF protein, where the linker is 16 amino acids and length and is comprised of these three amino acids (glycine, serine and alanine). Neither of these references describes a fusion that adds a free *cysteine* residue to EPO. Therefore, neither reference is relevant to the issue of introducing a free cysteine to EPO.

It is submitted that the addition of a cysteine at the C-terminus as presently claimed would be quite different than addition of an alanine, glycine or serine residue, because as previously discussed, cysteine is *unique* in its ability to disrupt protein structure, containing a highly reactive thiol group. Therefore, one cannot simply use examples of modification with

other amino acids to predict the effect of a cysteine modification at the same site. Indeed, Applicant has already provided evidence in the literature that modification of proteins with a non-cysteine amino acid versus a cysteine residue can result in proteins with widely different biological activities (*e.g.,* see previously submitted reference of Olins et al.). There is no evidence of record to support an argument that this observed fact would be expected to apply to a cysteine added to the C-terminus of a protein any less than it applies to a cysteine being substituted for another amino acid residue in the protein. The Examiner acknowledges that there is uncertainty in the art regarding the effect of different amino acids on protein activity, and accordingly, it is submitted that there is no reason the Examiner should not apply this evidence to C-terminal additions. Accordingly, the teachings of Weich and Mele are not adequate to support an argument that addition of a cysteine residue to the C-terminus of EPO would be predictably operable.

This argument is further supported by referring again to the references of Boissell and Quelle. The Examiner seems to accept that modification of the C-terminus of EPO may affect biological activity, but moves the argument to the use of C-terminal insertions and the references of Mele and Weich, which has been discussed above. However, a comparison of Boissell and Quelle also illustrates Applicant's position that the use of different amino acids to modify a protein can differently affect the biological activity of the protein. More specifically, each of Boissell and Quelle deleted the 4 amino acids from the C-terminus of EPO and then added additional amino acids to the C-terminus. In one case (Boissell), the protein was active, and in the other case, by adding a different combination of amino acids (Quelle), the protein was inactive. An important point of this comparison relevant to the Examiner's attempt to compare C-terminal additions of Mele and Weich to the presently claimed invention, is that it is not simply the deletion of the last 4 amino acids that yielded an inactive EPO protein, it is the specific sequence (or structure) of the added amino acids that determined whether the protein was active or inactive. In other words, these references again support Applicant's position that the use of different amino acids to modify a protein at the same position can result in dramatically different protein activities. Therefore, a teaching of the addition of a non-cysteine residue(s) to the C-terminus of EPO does <u>not</u> indicate or predict the result of adding a <u>cysteine</u> residue to the C-terminus of EPO.

Furthermore, the Examiner has provided one reference (Neilson et al.) that is an example of an EPO fusion that *lacks biological activity*, by his own admission. Despite the fact that the Examiner dismisses this reference on the basis that it is earlier than Weich and Mele, and because the fusion might not have activity due to the denaturing method, the cause of the inactivity is *not* resolved by Neilson or any other evidence of record (this is a different EPO fusion than that of Weich or Mele). Accordingly, one equally plausible reason for the inactivity remains the addition of another protein to the C-terminus of EPO that disrupted the structure of the protein. Comparison of this protein to the successful fusions of Weich and Mele is not sufficient to establish that it was a production step that caused activity problems in Neilson, because the proteins of Weich and Mele apply different amino acid additions to the C-terminus of EPO (*i.e.,* Neilson uses a different fusion to a different protein). As discussed above with respect to Boissell and Quelle, and as previously argued by reference to publications such as Olins (see Applicant's prior response), the use of different amino acids at the same position(s) can result in completely different activities in the resulting protein.

Moreover, with particular regard to the Examiner's argument that harsh denaturing conditions in Neilson could have been responsible for the fusion protein inactivity, thus dismissing this reference as less relevant than Weich or Mele, Applicants submit refer to the Examiner's cited references of WO 94/22466 and WO 94/12219 (discussed below), which describe production of some cysteine variants of IGF or IGFBP-1. These references use harsh denaturing conditions to produce their IGF/IGFBP-1 variants (see, *e.g.,* Example 3 of WO 94/12219 or page 45 of WO 94/22466, where denaturing conditions using 6M guanidine and 6mM DTT are described), yet are able to produce some biologically active cysteine variants of these proteins. Therefore, it is not clear as the Examiner asserts that those of skill in the art would have accepted that Neilson's failure to produce a biologically active fusion protein was most likely based on denaturing conditions. Instead, Applicants submit that an equally plausible reason was the C-terminal modification of EPO.

In summary, it is submitted that the Examiner has not established that one could predictably add a cysteine residue to the C-terminus of EPO and produce a biologically active protein. Indeed, the art as a whole supports Applicants' position that it would have been unpredictable prior to the present invention that addition of the cysteine to the C-terminus of EPO would be operable.

The Examiner also presented a second argument, which was made in response to Applicants' position that there is general unpredictability in the art regarding the effects of adding cysteine residues to proteins. The Examiner contends that both this problem, and the requirement for the C7-C161 bridge in EPO were known in the art at the time the application was filed. The Examiner contends that the art at the time of the invention provides teachings relating to the refolding of such cysteine-added proteins that overcome the problems of adding such free cysteines, referring to WO 94/22466 and WO 94/12219. Therefore, the Examiner asserts that the problems of adding a cysteine to a protein are overcome. Moreover, the Examiner asserts that there are no additional teachings in the present application for avoiding potentially detrimental effects of cysteine modification.

Applicants provide the following response to this argument and the attached publication, WO 95/32003. This argument is necessary and was not earlier presented, because the Examiner had not previously made reference to WO 94/22466 and WO 94/12219. Moreover, the provision of WO 95/32003 is an important part of Applicant's rebuttal of the argument based on newly cited WO 94/22466 and WO 94/12219, because it is a later filed publication by some of the same inventors, and it could not have been submitted earlier because the Examiner's argument had not yet been made. Therefore, Applicants require an opportunity to address these newly cited references and the Examiner's position that they support a rejection under 35 U.S.C. § 103. The Examiner is respectfully requested to fully consider this argument and attached reference.

In response to this portion of the Examiner's rejection, Applicant submits that the Examiner's argument does not address the merits of Applicant's position that it would have been unpredictable that addition of a cysteine residue to the end of the protein would have resulted in a stable and active protein. The fact that the prior art teaches *methods to produce* a protein with added cysteines once a suitable protein is provided is not sufficient to teach one of skill in the art *which* cysteine modifications can be successfully produced, which is provided by the present invention for EPO and the claimed EPO cysteine variant.

The Examiner's position is understood to be essentially that the prior art teaches methods to overcome issues with improper folding of proteins containing cysteine residues and therefore, the problem of having a cysteine residue in a protein is already overcome. Applicant submits that this is incorrect. Contrary to the Examiner's position that possession of a method of production is sufficient to overcome the problem of producing cysteine modified proteins, one

must also provide a cysteine modification that does not disrupt the activity of the protein (*i.e.,* the location of the modification *is* important, as argued above). Each of WO 94/22466 and WO 94/12219 in fact teach that the *location* of the added cysteine residue is important in producing a protein that folds properly and has good activity, which is further illustrated by WO 95/32003, enclosed herewith.

With respect to WO 94/22466, this publication teaches that cysteine modifications of IGFBP-1 may be possible between positions 60 and 180, with particularly preferred cites being residues 98 and 101 (see page 12), indicating that cysteine modification should be restricted to these sites (*i.e.,* the patent teaches that location of the cysteine modification is important). The publication does not disclose a particular method that is purported to overcome the problem of properly refolding proteins with cysteine modifications, as the Examiner seems to suggest, but rather simply refers to conventional methods in the art to produce the proteins (see page 13). WO 94/22466 produced only their preferred 98 and 101 cysteine variants, and so there is no evidence in this publication that selection of the particular residues to be modified was not primarily responsible for the successful production of biologically active muteins. The Examiner has provided no evidence that simply by following the method of protein production in WO 94/22466, any cysteine variant of any protein can be produced.

Referring to WO 94/12219, this publication also specifically directs where cysteine modifications can be made in IGF-1 (see page 8), and such positions *are fairly limited within the protein*, again illustrating that this publication teaches that *location* of the modification is important for producing a biologically active protein. Again, this reference simply refers to conventional methods for production of the protein (see page 9), illustrating that this publication does not teach a particular method to overcome the problem of properly refolding proteins with cysteine modifications. WO 94/12219 only describes results for two particular cysteine variants at positions 2 and 3 (although it discloses that a variant at position 1 was also made without providing activity data), and therefore, it is impossible to determine from this publication that it was a production method as implied by the Examiner, rather than the selection of particular cysteine modifications, that resulted in the successful production of IGF-1 variants. However, this question is readily answered by reviewing a subsequent patent publication by the same group of inventors.

Specifically, enclosed herewith is WO 95/32003, which is a subsequent filing to WO 94/12219, and which contains the same data as 94/12219, plus additional data on several other cysteine variants of IGF-1. Referring to Examples 3-5 of WO 95/32003, certain of the IGF-I cysteine muteins folded properly and had good activities, whereas others, refolded *using the same method*, refolded poorly and had low activities, some having incorrect or mixed disulfides. Moreover, certain variants, such as IGF-1 with a cysteine at position 3, were active in some conditions but had incorrect disulfides. The last paragraph of Example 3 and the first paragraphs of Example 4, for instance, show that many of the cysteine variants misfolded and contained incorrect disulfide linkages. Example 5 and Table 5 show that many of the cysteine variants, all produced using the *same method*, had poor bioactivities as compared to wild-type protein. Indeed, it appears that only three of the several different cysteine variants produced had sufficient biological activity to merit *in vivo* study. Therefore, the Examiner is not correct to conclude that the teachings in the prior art adequately enable those of ordinary skill in the art to make any cysteine modified protein, including the claimed cysteine modified proteins. Clearly, the selection of the location of the cysteine modification is important, and the cited references fail to teach or suggest the claimed invention.

The Examiner's argument that the prior art was aware of the requirement for the C7-C161 bridge being essential for EPO activity seems to support Applicant's position that a cysteine residue added following R166, being in close proximity to C161, would be expected to potentially interfere with proper formation of the critical C7-C161 disulfide bond. Applicant has previously cited Elliot et al. for teaching that disruption of the C7-C161 disulfide bond in EPO by substituting Ser for C7 or Ser for C161 results in EPO proteins possessing severely altered structures and possessing less than 2% of wild type EPO bioactivity. Accordingly, it was argued that the cysteine residue added by the inventor following R166 is in close proximity to C161 and therefore, based on Elliot et al., one of skill in the art might expect that this insertion would interfere with proper formation of the critical C7-C161 disulfide bond. The Examiner appears to assert that methods of producing the protein can overcome such issues, but it is submitted that if the addition of a C-terminal cysteine disrupted the C7-C161 disulfide bond, it is unclear how a method of refolding the protein as taught by WO 94/22466 and WO 94/12219 would resolve the problem, since it would be the structural modification that disrupts the protein and its bioactivity. Moreover, as discussed above, the methods of WO 94/22466 and WO 94/12219, as illustrated by

WO 95/32003, fail to produce biologically active proteins with respect to a variety of cysteine modified proteins.

Finally, in response to the Examiner's assertion that there are no additional teachings in the present application for avoiding potentially detrimental effects of adding a cysteine to a protein, this is also incorrect. The present specification, in contrast to providing a method to attempt to address the problem of producing cysteine variants, teaches *where* to add cysteine residues to EPO to avoid the need to rely on special methods of purification to obtain a biologically active protein. The present specification provides rules and clear teachings regarding where EPO will tolerate cysteine modifications and has shown through experimental evidence that the claimed invention operates as intended.

In summary, Applicant submits that the combination of references fails to teach or suggest the claimed EPO cysteine variant and fails to provide one of ordinary skill in the art with any guidance or expectation of success at being able to produce the claimed EPO cysteine variant. In view of the foregoing remarks, the Examiner is respectfully requested to withdraw the rejection of Claims 25 and 44-46 under 35 U.S.C. § 103.

This response is believed to address all remaining concerns as set forth in the May 10 Office Action, and it is submitted that the claims are in a condition for allowance. The Examiner is encouraged to contact the below-named agent at (303) 863-9700 to discuss any further issues and to expedite allowance of the claims.

Respectfully submitted,

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Date: September 18, 2007

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 47/48 — A1

(11) International Publication Number: **WO 95/32003**

(43) International Publication Date: 30 November 1995 (30.11.95)

(21) International Application Number: PCT/US95/06540

(22) International Filing Date: 24 May 1995 (24.05.95)

(30) Priority Data:
08/248,273 24 May 1994 (24.05.94) US

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(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: MODIFIED INSULIN-LIKE GROWTH FACTORS

(57) Abstract

Modified forms of insulin-like growth factor (IGF) are provided which demonstrate improved pharmacological and biological properties. These modifications include IGF muteins produced by substituting or adding a cysteine in the amino acid sequence of native IGF as well as such muteins attached to polyethylene glycol (PEG) at the free cysteine site. The present invention further provides methods of making such modified forms. The IGF-PEG conjugates can be formulated into pharmaceutical compositions and used for the therapeutic treatment of IGF associated conditions.

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MODIFIED INSULIN-LIKE GROWTH FACTORS

Field of the Invention

This invention relates to the modification of polypeptides, and more particularly to the modification of insulin-like growth factors and to methods of making and using such modified polypeptides.

Background of the Invention

The insulin gene family, comprised of insulin, relaxin, insulin-like growth factors 1 and 2, and possibly the beta subunit of 7S nerve growth factor, represents a group of structurally related polypeptides whose biological functions have diverged as reported in Dull, et al., Nature 310:777-781 (1984).

Insulin-like growth factors 1 and 2 (IGF-1 and IGF-2) are about 7-8 kilodalton proteins that are structurally related to each other and to insulin. IGF-1 and IGF-2 share about 70% amino acid identity with each other and about 30% amino acid identity with insulin. IGF-1 and IGF-2 are believed to have related tertiary structures as reported in PCT Application Publication No. WO 90/00569, published on January 25, 1990. The structural similarity between IGF-1 and IGF-2 permits both to bind to IGF receptors. Two IGF receptors are known to exist. IGF-1 and IGF-2 bind to the IGF type I receptor, while insulin binds with less affinity to this receptor. The type I receptor preferentially binds IGF-1 and is believed to transduce the mitogenic effects of IGF-1 and IGF-2. IGF-2 binds to the type I receptor with a 10-fold lower affinity than IGF-1. The second or type II IGF receptor preferentially binds IGF-2. Receptor binding is believed to be necessary for the biological activities of IGF-1 and IGF-2.

IGF-1 and IGF-2 are mitogenic for a large number of cell types, including fibroblasts, keratinocytes, endothelial cells and osteoblasts (bone-forming cells). IGF-1 and IGF-2 also stimulate differentiation of many cell types, e.g., synthesis and secretion of collagens by osteoblasts. IGF-1 and IGF-2 exert their mitogenic and cell differentiating effects by binding to the specific IGF cell surface receptors. IGF-1 also has been shown to inhibit protein catabolism in vivo, stimulate glucose uptake by cells and to promote survival of isolated neurons in culture. These properties have led to IGF-1 being tested as a therapeutic agent for a variety of disease indications as reported in Froesch et al., Trends

in Endocrinology and Metabolism, 254-260 (May/June 1990) and Cotterill, Clinical Endocrinology, 37:11-16 (1992). In addition to specific cell surface receptors, there exist at least six distinct IGF binding proteins (IGFBP-1 through IGFBP-6) that circulate throughout the body. These proteins bind IGF-1 and IGF-2 with high affinity. The binding of IGF-1 and IGF-2 to binding proteins reduces the action of these IGFs on cells by preventing their interaction with cell surface IGF receptors. IGF binding proteins, particularly IGFBP-3, also function to prolong the circulating half-lives of IGF-1 and IGF-2 in the blood stream. In the absence of IGF binding proteins, the half-life of IGF-1 in blood is less than 10 minutes. In contrast, when IGF-1 is bound to IGFBP-3, its half-life in blood is lengthened to about 8 hours. The circulating half-life of IGF-1 bound to the other smaller binding proteins is about 30 minutes as reported in Davis et al., J. of Endocrinology, 123:469-475 (1989); Guler et al., Acta Endocrinologica, 121:753-758 (1989); and Hodgkinson et al., J. of Endocrinology, 123:461-468 (1989). When IGF is bound to binding proteins, it is unable to bind to the IGF receptors and is therefore, no longer active in the body. Decreased affinity to binding proteins allows more of the IGF to be active in the body. Situations when this decreased affinity to binding proteins may be useful include, for example, cachexia, osteoporosis, and peripheral neuropathies.

Furthermore, the therapeutic utility of IGF can be modified by the presence or absence of these IGF binding proteins, which may potentiate or inhibit the beneficial effects of IGF. The levels of certain IGF binding proteins can vary greatly, depending upon the disease state. For example, IGFBP-1 levels are very high in diabetes patients, whereas they are nearly undetectable in normal patients as reported in Brismar et al., J. of Endocrinological Investigation, 11:599-602 (1988); Suikkari et al., J. of Clinical Endocrinology and Metabolism, 66:266-273 (1988); and Unterman et al., Biochem. Biophys. Res. Comm., 163:882-887 (1989). IGFBP-3 levels are reduced in severely ill patients such as those that have undergone major surgery as reported in Davies et al., J. Endocrinology, 130:469-473 (1991); Davenport et al., J. Clin. Endocrin. Metab., 75:590-595 (1992). The reduced levels of IGFBP-3, and consequent shorter circulating half-life of IGF-1, may contribute to the cachexia (weight loss) seen in these patients.

Insulin-like growth factor 1 (IGF-1), also known as somatomedin C, has long been studied for its role in the growth of various tissues. Its role as a useful therapeutic agent for several disease conditions has been suggested. Significantly reduced levels of IGF-1

were found in 23 patients with varying extent and severity of burns as reported in Moller et al., Burns, 17(4):279-281 (1991). A marked rise in serum type III procollagen, a marker of bone formation, occurred after one week of administration of recombinantly produced IGF-1 to patients with dwarfism otherwise non-responsive to growth hormone as reported in Laron et al., Clinical Endocrinology, 35: 145-150 (1991). The effects of the infusion of IGF-1 in a child with Laron Dwarfism is described in Walker et al., The New England Journal of Medicine, 324(21):1483-1488 (1991). Increased weight gain, nitrogen retention and muscle protein synthesis following treatment of diabetic rats with IGF-1 or IGF-1 having a deletion of the first three amino acids ordinarily found in IGF-1 (referred to as "(des1-3)IGF-1") were demonstrated in Tomas et al., Biochem. J., 276:547-554 (1991). Growth restoration in insulin-deficient diabetic rats by administration of recombinantly produced human IGF-1 is reported in Scheiwiller et al., Nature, 323:169 (1986). IGF-1 and (des1-3)IGF-1 enhance growth in rats after gut resection, as reported in Lemmey et al., Am. J. Physiol., 260 (Endocrinol. Metab. 23) E213-E219 (1991). A combination of platelet-derived growth factor and insulin-like growth factors, including IGF-1, enhanced periodontal regeneration in beagle dogs as reported by Lynch et al., J. Clin. Periodontal, 16:545-548 (1989). The synergistic effects of platelet-derived growth factor and IGF-1 in wound healing are reported in Lynch et al., Proc. Natl. Acad. Sci., 84:7696-7700 (1987). The effects of IGF-1 and growth hormone on longitudinal bone growth in vitro are set forth in Scheven and Hamilton, Acta Endocrinologica (Copenhagen) 124:602-607 (1991). In vivo actions of IGF-1 on bone formation and resorption in rats are shown in Spencer et al., Bone, 12:21-26 (1991). The use of IGF-1 and IGF-2 for enhancing the survival of non-mitotic, cholinergic neuronal cells in a mammal is described in U.S. Patent 5,093,317 to Lewis et al. In addition, PCT Application Publication No. WO 92/11865 published on July 23, 1992, describes the use of IGF-1 for the treatment of cardiac disorders.

Various modifications to the naturally occurring or wild-type IGF-1 have been described. For example, the naturally occurring variant of IGF-1 missing from the first three N-terminal amino acids, (des1-3)IGF-1, was discovered in cerebral spinal fluid and in colostrum as reported in Sara, et al., Proc.Natl.Acad.Sci., 83:4904-4907 (1986) and Francis et al., Biochemical Journal, 251:95-103 (1988). In vitro studies have shown that this variant is equipotent to IGF-1 in binding to IGF cell surface receptors and in stimulating cell mitogenesis. Thus, the first three amino acids of IGF-1 appear to be

nonessential for the binding of IGF-1 to its specific cell surface receptors. (Des1-3)IGF-1 was found to have greatly reduced affinity (100-fold less) for certain IGF binding proteins, specifically IGFBP-1 and IGFBP-2, as reported in Forbes et al., Biochem. Biophys. Res. Comm., 157:196-202 (1988); and Carlsson-Skwirut et al., Biochim. Biophys. Acta, 1101:192-197 (1989). The binding of IGFBP-3 to (des1-3)IGF-1 also is affected, being reduced by two to three fold. Animal studies have shown that (des1-3)IGF-1, when given by continual subcutaneous infusion, is more potent than IGF-1 in stimulating a number of anabolic functions, such as growth, reported in Cascieri et al., J. Endocrinology, 123:373-381 (1988) and Gillespie et al., J. Endocrinology, 127:401-405 (1990). The enhanced properties of (des1-3)IGF-1 are believed to result from its reduced affinity for IGF binding proteins, reported in Carlsson-Skwirut et al., Biochim. Biophys. Acta, 1101:192-197 (1989). The reduced affinity of (des1-3)IGF-1 for IGF binding proteins results in (des1-3)IGF-1 having a shorter circulating half-life than wild type IGF-1 as reported in Cascieri et al., J. Endocrinology, 123:373-381 (1988). Therefore, (des1-3)IGF-1 must be administered by continual infusion or by multiple daily injections in order to effect its enhanced potency.

PCT Application Publication No. WO 89/05822 published on June 29, 1989, describes other modifications of IGF-1. This application describes substituting the third amino acid from the N-terminal end of the naturally occurring IGF-1 with glycine, glutamine, leucine, arginine or lysine to form IGF-1 muteins. This reference however does not teach replacing the third amino acid with cysteine.

The potential therapeutic usefulness of IGF-1 and (des1-3) IGF-1 is limited by their short circulating half-lives to situations when the proteins can be administered by continual infusion or by multiple daily injections to achieve their maximum therapeutic potential. As an example, Woodall et al., Horm. Metab. Res., 23: 581-584 (1991), reports that the same total dose of IGF-1 administered four times daily by subcutaneous injection was far superior in stimulating growth in mutant lit/lit mice (growth hormone deficient mice) than was the same total dose administered once daily.

There are many cases in which it would be preferable to administer IGF-1 in a single once-a-day injection or in a single injection given once every several days. For injectable drugs, patient compliance is expected to be higher for drugs that can be administered once a day rather than several times a day. In order for IGF-1 to be

therapeutically effective when given once a day or once every few days, methods must be developed to increase its circulating half-life.

Increasing the molecular weight of a protein by covalently bonding an inert polymer chain such as polyethylene glycol (PEG) to the protein is known to increase the circulating half-life of the protein in the body. See, for example, Davis et al, Biomedical Polymers: Polymeric Materials and Pharmaceuticals for Biomedical Use, p. 441-451 (1980). However, since multiple PEG molecules can bind to each protein molecule, and because there are typically a large number of sites on each protein molecule suitable for binding to several PEG molecules using known methods, there has been little success in attaching PEG to yield homogeneous reaction products. See Goodson et al, Biotechnology, 8:343 (1990), and U.S. Patent 4,904,584. This lack of site attachment specificity can give rise to a number of problems, including loss of activity of the protein.

Thus, a need exists for prolonging the circulating half life of IGF without compromising its usefulness as a therapeutic agent. The present invention satisfies this need by increasing the molecular weight of the IGF. This is accomplished by providing muteins of IGF suitable for thiol-specific attachment of PEG to IGF and PEG conjugates formed from such muteins.

Summary of the Invention

The invention relates to various modified forms of IGF. One type of modified IGF, referred to as muteins, is produced by replacing cysteine residues for specific amino acids in the wild type molecule, or by inserting cysteine residues adjacent to or between amino acids in the wild type molecule. Cysteine residues which are not involved in intramolecular disulfide bonds are considered to be "free". Cysteine residues can be substituted or inserted in regions of the IGF molecule that are exposed on the protein's surface. For example, the non-native cysteine can be inserted or substituted within the first or last twenty amino acids of wild-type IGF-1. This non-native cysteine is expected to be free and to serve as the attachment site for the polyethylene glycol (PEG) molecules to IGF, resulting in PEGylated molecules. In some cases, however, during refolding of the mutein or during reduction of the mutein before reaction with PEG, the non-native cysteine can become involved in a disulfide bond and thereby free a native cysteine for PEGylation. Attachment of the PEG

molecule to a mutein creates a further modified form of IGF, or IGF-PEG conjugate of defined structure, where the PEG is attached to the IGF at a free cysteine residue.

Thus, the present invention is directed to a polyethylene glycol (PEG) conjugate comprising PEG and a mutein of IGF, and particularly IGF-1, where the PEG is attached to the mutein at a non-native cysteine. PEG can be attached to the free cysteine through a thiol-specific activating group including, for example, maleimide, sulfhydryl, thiol, triflate, tresylate, aziridine, exirane and 5-pyridyl. A suitable PEG can have a molecular weight of 5 kDa, 8.5 kDa, 10 kDa or 20 kDa. The PEG conjugate of the present invention can also contain a second protein to form a dumbbell. Methods of making the PEG conjugates are also provided.

Moreover, PEG is known to increase the circulating half-life of a protein in the body. Thus, IGF can be administered to patients less frequently with equal or better effectiveness than in the past.

The present invention is further directed to muteins of IGF particularly those having a non-native cysteine in the N-terminal or C-terminal region of the mutein. The muteins can be produced by recombinant methods.

Also provided in the present invention are pharmaceutical compositions comprising the IGF-PEG conjugate and methods of using the IGF-PEG conjugate to treat a patient having or potentially having an IGF associated condition.

Detailed Description of the Invention

The present invention is directed to modified forms of insulin-like growth factors (IGF) that provide beneficial properties not exhibited by wild-type IGF. The modified forms of IGF include muteins of these growth factors, containing at least one free cysteine. Conjugates containing the IGF muteins attached to polyethylene glycol (PEG) are also considered modified forms of IGF.

Terms used throughout the specification and claims are defined as follows:

The term "IGF" refers to any polypeptide that binds to the IGF type I Receptor, including, for example, IGF-1, IGF-2, (des1-3)IGF-1, (R3)IGF-1 which is a mutein having a non-native arginine at residue number 3, and insulin. This hormone family is described in Blundell and Humbel, Nature, 287:781-787 (1980). Due to this common receptor

binding, the teachings of the present invention which are described with respect to IGF-1 are intended to encompass IGF-2, des(1-3) IGF-1, (R3)IGF-1, and insulin also.

The term "wild type IGF" refers to the unmodified or naturally occurring IGF. This term is used interchangeably with "IGF," "naturally occurring IGF," or "native IGF." The term "wild type IGF" also refers to native IGF to which a methionine residue has been added at the N-terminus.

The term "wild type IGF-1" refers to the unmodified or naturally-occurring 70 amino acid form of IGF-1. This term is used interchangeably with "IGF-1," "naturally occurring IGF-1," and "native IGF-1." The term "wild type IGF-1" also refers to native IGF-1 to which a methionine residue has been added at the N-terminus.

The term "non-native" refers to that which is not found in the native molecule.

The term "IGF-PEG conjugate" refers to an IGF molecule attached to a polyethylene glycol molecule. This is also referred to as a "Peg conjugate".

The term "N-terminal region" refers to approximately the first twenty amino acids from the N-terminus of IGF or an IGF mutein, and up to twelve amino acids preceding the first amino acid of the N-terminus of IGF.

The term "N-terminus" refers to the first amino acid at the N-terminal region in the sequence of wild-type IGF, for example, glycine in IGF-1.

The term "C-terminal region" refers to aproximately the last twenty amino acids from the C-terminus of IGF or an IGF mutein and up to twelve amino acids following the last amino acid of the C-terminus of IGF.

The term "C-terminus" refers to the last amino acid at the C-terminal region in the sequence of wild-type IGF, for example, alanine in IGF-1.

The term "mutein" refers to a modified form of IGF, which has been modified to contain a non-native cysteine.

The term "retain biological activity" refers to having at least 10% of the mitogenic activity of wild type recombinant IGF as measured by the relative amount of ^{3}H-thymidine incorporated into UMR106 rat osteosarcoma cells, in the absence of IGF binding protein-1, using the assay described herein. The muteins and the conjugates of the present invention retain biological activity.

The term "activating group" refers to a site on the PEG molecule which attaches to the mutein.

The term "pharmaceutically acceptable carrier" refers to a physiologically-compatible, aqueous or non-aqueous solvent.

The term "free cysteine" refers to any cysteine residue not involved in an intramolecular disulfide bond.

The term "IGF associated condition" refers to an existing or potential adverse physiological condition which results from an over-production or underproduction of IGF, IGF binding protein or IGF receptor, inappropriate or inadequate binding of IGF to binding proteins or receptors and any disease in which IGF administration alleviates disease symptoms. An IGF associated condition also refers to a condition in which administration of IGF to a normal patient has a desired effect.

The term "patient" refers to any human or animal in need of treatment for an IGF associated condition.

The IGF muteins of the present invention are produced by modifying wild-type IGF, particularly at the N-terminal or C-terminal region of the protein. Such modifications can be substitutions or additions of at least one cysteine residue. An IGF mutein can be produced by replacing a specific amino acid with a cysteine, such as, for example, substituting one of the first or last four amino acids of IGF-1 with a cysteine residue. The amino acid sequence of wild type IGF-1 starting from the N-terminal end is: G P E T L C G A E L V D A L Q F V C G D R G F Y F N K P T G Y G S S S R R A P Q T G I V D E C C F R S C D L R R L E M Y C A P L K P A K S A (SEQ ID NO. 1).

Other modifications include, for example, adding at least one cysteine residue in front of the first or after the last amino acid of IGF. For example, a cysteine residue can be inserted in front of and adjacent to the first amino acid of IGF. For muteins produced by E. coli, the non-native cysteine can appear between Met and the first amino acid of IGF. A free cysteine residue can also appear in a group of about twelve or less amino acids inserted before the first or after the last amino acid of IGF to form a longer IGF mutein.

In particularly useful embodiments, the non-native cysteine residues are located in regions of the IGF-1 molecule exposed to the protein's surface. The N-terminal region, for example, is involved in the binding of the IGF to binding proteins, but is not involved in binding of IGF to cell surface IGF receptors.

An IGF-1 mutein of the present invention is also referred to as "a cysteine mutein of IGF-1." The non-native cysteine residue can act as the attachment site for covalent

linkage of the activating group on the polyethylene glycol. Alternatively, the non-native cysteine can become involved in disulfide bonding thereby freeing a native cysteine residue for thiol-specific attachment to PEG. The newly created molecule comprising the cysteine mutein of IGF with the PEG attached is referred to as a "PEG conjugate of IGF".

The IGF muteins of the present invention can be prepared by methods well known to one skilled in the art. Such methods include mutagenic techniques for replacement or insertion of an amino acid residue, as described, for example, in U.S. Patent 4,518,584, incorporated herein by reference. The muteins produced by mutagenic techniques can then be expressed as recombinant products according to procedures known to those skilled in the art. The muteins can alternatively be synthesized by conventional methods known in the art. The IGF muteins can also be prepared according to the methods and techniques described in the examples set forth below.

The present invention also provides IGF-PEG conjugates and methods of making such conjugates by attaching the IGF muteins to polyethylene glycol to increase the circulating half-life of the molecule in the body as well as decrease its affinity to IGF binding proteins.

In the present invention long chain polymer units of polyethylene glycol (PEG) are bonded to the mutein via a covalent attachment to the sulfhydryl group of a free cysteine residue on the IGF mutein. Various PEG polymers with different molecular weights, 5.0 kDa (PEG_{5000}), 8.5 kDa (PEG_{8500}), 10 kDa ($PEG_{10,000}$), and 20 kDa ($PEG_{20,000}$) can be used to make the IGF-PEG conjugates. In order to obtain selectivity of reaction and homogenous reaction mixtures, it is useful to use functionalized polymer units that will react specifically with sulfhydryl or thiol groups. The functional or reactive group attached to the long chain polyethylene glycol polymer is the activating group to which the IGF mutein attaches at a free cysteine site. Useful activating groups include, for example, maleimide, sulfhydryl, thiol, triflate, tresylate, aziridine, exirane, or 5-pyridyl.

In another embodiment, polyethylene glycol (PEG) polymers containing two activating groups can be used to create "dumbbell" type molecules containing two IGF muteins attached to one molecule of PEG at each end of the PEG molecule. For example, PEG bis-malemide (a polyethylene glycol polymer containing a maleimide activating group on each end of the PEG molecule) can be used to create these "dumbbell" type molecules. These dumbbell molecules can also comprise a single IGF mutein covalently attached via

PEG to a second protein or peptide of different structure. The second protein or peptide can be, for example, a growth factor such as platelet-derived growth factor, or fibroblast growth factor.

One skilled in the art can readily determine the appropriate pH, concentration of protein, and ratio of protein:PEG necessary to produce a useful yield of either mono-pegylated IGF-1 (IGF-PEG), or dumbbell IGF-1 (IGF-PEG-IGF, IGF-PEG-PDGF, or IGF-PEG-FGF) using conventional methods known to one skilled in the art for making these determinations.

The invention present also includes pharmaceutical compositions. The IGF muteins and PEG conjugates can be in a pharmaceutically-acceptable carrier to form the pharmaceutical compositions of the present invention. The term "pharmaceutically acceptable carrier" as used herein means a non-toxic, generally inert vehicle for the active ingredient, which does not adversely affect the ingredient or the patient to whom the composition is administered. Suitable vehicles or carriers can be found in standard pharmaceutical texts; for example, in Remington's Pharmaceutical Sciences, 16th ed., Mack Publishing Co., Easton, PA (1980), incorporated herein by reference. Such carriers include, for example, aqueous solutions such as bicarbonate buffers, phosphate buffers, Ringer's solution and physiological saline. In addition, the carrier can contain other pharmaceutically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation.

The pharmaceutical compositions can be prepared by methods known in the art, including, by way of an example, the simple mixing of reagents. Those skilled in the art will know that the choice of the pharmaceutical carrier and the appropriate preparation of the composition depend on the intended use and mode of administration.

In one embodiment, it is envisioned that the carrier and the IGF mutein or conjugate constitutes a physiologically-compatible, slow-release formulation. The primary solvent in such a carrier can be either aqueous or non-aqueous in nature. In addition, the carrier can contain other pharmacologically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the carrier can contain still other pharmacologically-acceptable excipients for modifying or maintaining the stability, rate of dissolution, release, or absorption of the IGF mutein or conjugate. Such excipients are those substances usually

and customarily employed to formulate dosages for parenteral administration in either unit dose or multi-dose form.

Once the pharmaceutical composition has been formulated, it can be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or dehydrated or lyophilized powder. Such formulations may be stored either in a ready to use form or requiring reconstitution immediately prior to administration. The preferred storage of such formulations is at temperatures at least as low as 4°C and preferably at -70°C. It is also preferred that such formulations containing the IGF mutein or conjugate are stored and administered at or near physiological pH. It is presently believed that administration in a formulation at a high pH (i.e. greater than 8) or at a low pH (i.e. less than 5) is undesirable.

The manner of administering the formulations containing the IGF mutein or conjugate for systemic delivery can be via subcutaneous, intramuscular, intravenous, oral, intranasal, or vaginal or rectal suppository. Preferably the manner of administration of the formulations containing the IGF muteins or conjugates for local delivery is via intraarticular, intratracheal, or instillation or inhalations to the respiratory tract. In addition it may be desirable to administer the IGF muteins or conjugates to specified portions of the alimentary canal either by oral administration of the IGF muteins or conjugates in an appropriate formulation or device.

For oral administration, the the IGF muteins or conjugates are encapsulated. The encapsulated IGF muteins or conjugates may be formulated with or without pharmaceutically-acceptable carriers customarily used in the compounding of solid dosage forms. Preferably, the capsule is designed so that the active portion of the formulation is released at that point in the gastro-intestinal tract when bioavailability is maximized and pre-systemic degradation is minimized. Additional excipients may be included to facilitate absorption of the IGF muteins or conjugates. Diluents, flavorings, low melting point waxes, vegetable oils, lubricants, suspending agents, tablet disintegrating agents, and binders may also be employed.

Regardless of the manner of administration, the specific dose is calculated according to the approximate body weight of the patient. Other factors in determining the appropriate dosage can include the disease or condition to be treated or prevented, route of administration and the age, sex and medical condition of the pateint. In certain embodiments, the dosage and administration is designed to create a preselected

concentration range of the IGF muteins or conjugates in the patient's blood stream. It is believed that the maintenance of circulating concentrations of the IGF muteins or conjugates of less than 0.01 ng per ml of plasma may not be an effective composition, while the prolonged maintenance of circulating levels in excess of 100 μg per ml may have undesirable side effects. Further refinement of the calculations necessary to determine the appropriate dosage for treatment involving each of the above mentioned formulations is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed by them without undue experimentation, especially in light of the dosage information and assays disclosed herein. These dosages may be ascertained through use of the established assays for determining dosages utilized in conjunction with appropriate dose-response data.

It should be noted that the IGF mutein and conjugate pharmaceutical compositions described herein may be used for veterinary as well as human applications and that the term "patient" should not be construed in a limiting manner. In the case of veterinary applications, the dosage ranges should be the same as specified above.

The pharmaceutical composition of the present invention can be used to treat a patient having or potentially having an IGF associated condition. Some of these conditions can include, for example, dwarfism, diabetes, periodontal disease and osteoporosis. The pharmaceutical composition of the present invention can also be used to treat a condition in which administration of IGF to a normal patient has a desired effect; for example, using IGF-1 to enhance growth of a patient of normal stature.

The following examples are intended to illustrate the present invention and are not intended to be limiting.

EXAMPLE 1

A. Construction of the IGF-1 gene

The IGF-1 gene was assembled in two stages. Initially, the DNA sequence encoding IGF-1 was joined to DNA sequences encoding the secretory leader sequence of the E. coli OMP A protein (ompAL). This gene fusion was constructed in order to determine whether IGF-1 could be efficiently secreted from E. coli. A second construct, in which IGF-1 is expressed as an intracellular protein in E. coli, was created by deleting DNA sequences

encoding the OmpA leader sequence and replacing them with DNA sequences that allow intracellular expression of IGF-1.

B. Construction of the OmpAL-IGF-1 gene fusion

The four synthetic oligonucleotides labeled OmpA1U: 5'GATCCGATCGTGGAGGATGATTAAATGAAAAAGACAGCTATCGCGATCGCA3' (SEQ ID NO. 2), OmpA2U: 5'GTGGCACTGGCTGGTTTCGCTACCGTA GCGCAGGCCGCTCCGTGGCAGTGC3' (SEQ ID NO. 3), OmpA1L: 5'CAGTGC CACTGCGATCGCGATAGCTGTCTTTTTCATTTAATCATCCTCCACGATCG3' (SEQ ID NO. 4) and OmpA2L: 5'GCACTGCCACGGAGCGGCCTGCGCTAC GGTAGCGAAACCAGC3' (SEQ ID NO. 5), were annealed pairwise (1U + 1L and 2U + 2L) and the pairs ligated together. All four of these oligonucleotides were synthesized using DNA synthesizers purchased from Applied Biosystems (Models 391 and 380A). The ligation mixture was then digested with the restriction enzyme HaeIII. The resulting BamHI/HaeIII restriction fragment coding for a translational start signal and the first 21 amino acids of the ompA signal sequence was purified. This DNA fragment was mixed with BamHI + PstI-digested PUC18 DNA (commercially available from Boehringer Mannhein Biochemicals, Indianapolis, IN) and the two synthetic oligonucleotides [IGF-1 (1-14) U + L] 5'CCGGTCCGGAGACTCTGTGCGGCGCAGAACTGGTTGAC GCTCTGCA3' (SEQ ID NO. 6) and 5'GAGCGTCAACCAGTTCTGCGCCGC ACAGAGTCTCCGGACCGG3' (SEQ ID NO. 7) were ligated together. The ligation mixture was used to transform E. coli strain JM109 (commercially available from New England Biolabs, Beverly, MA) and individual colonies isolated. These plasmids (OmpALIGF-1pUC18) have a translational start signal followed by DNA sequences encoding the OmpA signal sequence and the first 14 amino acids of IGF-1.

An M13 phage containing DNA sequences encoding amino acids 15 through 70 of IGF-1 was created by ligating together the two complementary pairs of oligonucleotides (IGF1U + 1L and IGF2U + 2L) 5'GTTCGTATGCGGCGACCGTGGCTTC TACTTCAACAAACCGACTGGCTACGGTTCCAGCTCTCGTCGTGCACCGCAG ACTGGTATC3' (SEQ ID NO. 8) and 5'TTCGTCAACGATACCAGTCTGCGGTGC ACGACGAGAGCTGGAACCGTAGCCAGTCGGTTTGTTGAAGTAGAAGCCACG GTCGCCGCATACGAACTGCA3' (SEQ ID NO. 9) and cloning the DNA fragment into

PstI + HindIII-digested M13 mp19 DNA (commercially available from New England Biolabs, Beverly, MA). Double-stranded DNA was purified from a phage clone and the PstI/HindIII fragment encoding amino acids 15-70 of the IGF-1 protein were isolated. This DNA fragment was ligated together with PstI + HindIII-digested plasmid OmpALIGF-1pUC18 DNA and used to transform E. coli strain JM107 (commercially available from GIBCO BRL, Gaithersburg, MD). The BamHI/HindIII fragment containing the IGF-1 gene fused to the OmpAL sequence was isolated and cloned into the BamHI + HindIII generated site of plasmid pT3XI-2 (described in PCT Application publication WO 91/08285 published on June 13, 1991). The completed plasmid containing the ompAL-IGF-1 gene fusion is called pT3XI-2 ø10C(TC3)ompALIGF-1.

C. Construction of the Methionyl-IGF-1 gene

The BamHI/HindIII fragment containing the OmpAL-IGF-1 gene fusion described above was purified from plasmid pT3XI-2ø10C(TC3)ompALIGF-1 and digested with HinfI. The approximate 200 bp HinfI/HindIII DNA fragment was mixed with the annealed, complementary synthetic oligonucleotides (MetIGF1U + 1L) 5'GATCCGATCGT GGAGGATGATTAAATGGCCGGTCCGGAG3' (SEQ ID NO. 10) and 5'AGT CTCCGGACCGGCCATTTAATCATCCTCCACGATCG3' (SEQ ID NO. 11) and ligated with BamHI + HindIII-digested plasmid pT3XI2 DNA, and used to transform E. coli JM107. The completed plasmid construct is called ø10C(TC3)IGF-1pT3XI-2 and contains an extra alanine residue in between the initiator methionine and the beginning of the IGF-1 sequence. The BamHI/HindIII fragment containing the mutant IGF-1 gene was isolated and ligated into the BamHI + HindIII generated site of plasmid pT5T (described in Nature, Vol. 343, No. 6256, pp. 341-346, 1990). The ligation mixture was used to transform E. coli BL21/DE3 described in US Patent 4,952,496 and the resulting individual colonies were isolated. This construct was named ø10C(TC3)IGF-1pT5T.

The extra alanine codon was removed by in vitro mutagenesis. In vitro mutagenesis was performed using a Muta-Gene kit purchased from Bio-Rad Laboratories (Richmond, CA). The mutagenesis procedure followed was essentially that described in the instructions that accompany the kit. Plasmid ø10C(TC3)IGF-1pT3XI-2 was digested with BamHI + HindIII and the _200 bp DNA fragment containing the mutant IGF-1 gene was purified and cloned into the BamHI and HindIII sites of plasmid M13 mp19.

Uracil-containing single-stranded template DNA was prepared following propagation of the phage in E. coli strain CJ236 (supplied with Muta-Gene Kit purchased from Bio-Rad Laboratories, Richmond, CA). The oligonucleotide used for mutagenesis had the sequence: 5'GATGATTAAATGGGTCCGGAGACT3' (SEQ ID NO. 12). The mutagenesis reaction product was used to transform E. coli strain JM109 and individual plaques picked.

Double-stranded replicative form DNA from individual phages was isolated, digested with BamHI + HindIII and the _200 bp fragment containing the IGF-1 gene purified. The purified DNA was cloned into the BamHI + HindIII generated site of plasmid pT5T and used to transform E. coli strain BL21/DE3. One bacterial colony with the correct plasmid was named ø10(TC3)mutIGF-1pT5T. Several isolates were sequenced, and all were correct.

EXAMPLE 2

Construction of IGF-1 Muteins

Several muteins of IGF-1 were constructed. Three of the muteins replaced each of the first three amino acids of IGF-1 with a cysteine residue. These muteins are referred to as C1, C2, and C3, respectively. A fourth mutein introduced a cysteine residue between the N-terminal methionine residue and the first amino acid of IGF-1. This mutein is referred to as -1C.

The -1C, C1, C2 and C3 muteins of IGF-1 were made using the polymerase chain reaction (PCR) technique as described below. The starting plasmid used for the mutagenesis experiments was ø10(TC3)mutIGF-1pT5T, which is described in Example 1. This plasmid contains DNA sequences encoding an initiator methionine followed by the sequence of the natural human IGF-1 protein. Mutant IGF-1 DNA sequences were amplified from this gene using a 5' mutagenesis oligonucleotide that contained the desired mutation and a 3' oligonucleotide of correct sequence. The 5' mutagenesis oligonucleotides were designed so that they incorporated the first methionine of the gene as part of an Nde I restriction enzyme cleavage site (CATATG). Each mutagenesis oligonucleotide contained the desired mutation followed by 15 to 21 nucleotides that were a perfect match to the IGF-1 gene sequences in plasmid ø10(TC3)mutIGF-1pT5T. The 3' oligonucleotide was 25 nucleotides long and was designed to encode the last 6 codons of IGF-1 and to contain the Hind III site that follows the stop codon.

In addition, a wild type clone was made in the same manner using the NdeI site of pT5T (described above) as the first methionine. This wild type clone is designated 85p-11. The oligonucleotides used to construct the -1C, C1, C2 and C3 muteins and the 85p-11 wild type clone are set forth in Table 1.

TABLE 1

mutein	a.a. sequence	oligo	sequence
wild type	M G P E T L C...	IGF(85p)28	5'GGGCATATGGGTCCGGAGACTCTGTGCG3'
-1C	M C G P E T L C...	IGF(-1Cp)33	5'GGGCATATGTGTGGTCCGGAGACTCTGTGCGGC3'
C1	M C P E T L C...	IGF(C1p)33	5'GGGCATATGTGCCCGGAGACTCTGTGCGGCGCA3'
C2	M G C E T L C...	IGF(C2p)31	5'GGGCATATGGGTTGTGAGACTCTGTGCGGCG3'
C3	M G P C T L C...	IGF(C3p)33	5'GGGCATATGGGTCCGTGCACTCTGTGCGGCGCA3'
3' oligo (for all of the above muteins)		IGF(262p)25	5'CCCAAGCTTAAGCGCTTTTAGCCGG3'

(overlaps with clone ø10(TC3)mutIGF-1pT5T are underlined)

(SEQ ID NOS. 13 through 18 respectively)

Polymerase chain reaction (PCR) was performed in 100ul reactions containing 20 mM Tris pH 8.8, 10 mM KCl, 6 mM (NH4)2SO4, 1.5 mM MgCl2, 0.1% Triton X-100 using 20 pmole of each oligo and approximately 1ng of plasmid ø10(TC3)mutIGF-1pT5T as template DNA. 0.5ul (1.25 units) of Pfu polymerase (Stratagene, San Diego, CA) was added after the first denaturation step with the tubes held at 65°C. The reactions were overlayed with 2 drops of mineral oil at that time. The reactions were cycled 30 times for 1 min. at 95°C, 1 min. at 65°C, and 1 min. at 72°C in an Ericomp Twinblock™ thermal cycler (Ericomp, San Diego, CA). After the last cycle the reactions were held at 72oC for 10 minutes.

After PCR, 80ul of the reactions were phenol extracted one time then ethanol precipitated. The precipitated DNA was resuspended in 80ul of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and 20ul was digested with Nde I and Hind III and electrophoresed on a 1.5% agarose gel. The amplified DNA bands that ran at approximately 210bp were eluted using NA45 paper (Schleicher and Schuell, Keene, NH) according to the manufacturer's instructions. The eluted DNA was resuspended in 20ul of TE buffer and 2ul was ligated to gel-purified Nde I and Hind III digested plasmid pT5T in a volume of 20ul. Plasmid pT5T is described in Example 1. The ligation reactions were used to transform E. coli strain BL21/DE3 and colonies selected on LB agar plates containing 50ug/ml of ampicillin. Mini plasmid DNA preps were made from several colonies from the transformation plates. The DNAs were digested with Eco RV and Hind III to determine which transformants contained IGF-1 DNA inserts. Plasmids containing IGF-1 DNA inserts were sequenced to verify that the inserts were correctly mutated (the entire IGF gene was sequenced for each mutein).

Preliminary growth studies were performed by growing a representative transformant for each mutein in Luria Broth + 12ug/ml tetracycline to an approximate OD_{600} of about 1.0. Isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to 1 mM to induce expression of T7 polymerase and the subsequent transcription and translation of the IGF muteins. Approximately 0.1 OD unit of cells were lysed in SDS sample buffer by boiling for two minutes and electrophoresed on a 16% polyacrylamide SDS gel. The gel was stained with Coomassie blue. IGF-1 protein bands of the expected size, which is approximately 7-8 kDa, were observed in the lanes loaded with induced cells for each mutein as well as for the wild-type control.

Muteins in which cysteine was substituted for the amino acids at residues 11, 12, 15, 16, 55, 64, 65, 67 and 69 of the wild type IGF-1 were also prepared using PCR. The PCR template was either the full-length plasmid from clone 85p-11 or the NdeI-HindIII fragment from clone 85p-11 containing the wild type IGF-I gene.

For muteins C11, C12, C15 and C16, 5' oligonucleotides were synthesized that incorporated the first methionine as part of the NdeI site (CATATG) and each contained the mutation desired followed by 21-24 nucleotides that were a perfect match to clone 85p-11. These oligonucleotides are set forth in Table 2. For the 3' end of the gene, a 25-mer oligonucleotide (IGF(262p)25) that matched the last 6 codons of IGF-I plus the HindIII site following the stop codon was used. IGF(262p)25 is set forth in Table 1.

TABLE 2

3' oligo for the muteins below		IGF(262p)25	5'CCCAAGCTTAAGCGCTTTTAGCCGG3'
mutein	a.a. sequence	oligo	sequence
C11	...A E L C...	IGF(C11)S-66	5'GGGCATATGGGTCCGGAGACTCTGTGCGGCGCAGAACTGTGCGACGCTCTGCAGTTCGTATGCGGC3'
C12	...A E L V C...	IGF(C12)S-69	5'GGGCATATGGGTCCGGAGACTCTGTGCGGCGCAGAACTGGTTTGCGCTCTGCAGTTCGTATGCGGCGAC3'
C15	...A E L V D A L C...	IGF(C15)77	5'GGGCATATGGGTCCGGAGACTCTGTGCGGCGCAGAACTGGTTGACGCTCTGTGTTTCGTATGCGGCGACCGTGGCTT3'
C16	...A E L V D A L Q C...	IGF(C16)78	5'GGGCATATGGGTCCGGAGACTCTGTGCGGCGCAGAACTGGTTGACGCTCTGCAGTGCGTATGCGGCGACCGTGGCTTC3'

(overlaps with IGF clone 85p-11 are underlined)

(SEQ ID NOS. 18 through 22 respectively; The 3' oligo is SEQ ID NO. 18)

For muteins C55, C64, C65, C67 and C69, 3' oligonucleotides that incorporated the HindIII site (AAGCTT), the stop codon (TAA) and containing the mutation desired followed by 21-27 nucleotides that were a perfect match to clone 85p-11 were synthesized. For the 5' end of the gene, a 28-mer oligonucleotide (IGF(85p)28) that matched the first 7 codons of IGF-I plus the initiation codon for methionine incorporated into an NdeI site was used. These oligonucleotides are set forth in Table 3.

TABLE 3

mutein	a.a. sequence	oligo	sequence
C55	...C D L C...	IGF(C55)81	5'CCCAAGCTTAAGCGCTTTTAGCCGGTTTCAGCGGAGCGCAGTACATTTCCAGACGGCACAGGTCGCAAGAACGGAAGCAGC3'
C64	...Y C A P C...	IGF(C64)AS-58	5'CCCAAGCTTAAGCGCTTTTAGCCGGTTTGCACGGAGCGCAGTACATTTCCAGACGGCG3'
C65	...Y C A P L C...	IGF(C65)AS-55	5'CCCAAGCTTAAGCGCTTTTAGCCGGGCACAGCGGAGCGCAGTACATTTCCAGACG3'
C67	...P L K P C...	IGF(C67)AS-49	5'CCCAAGCTTAAGCGCTTTTGCACGGTTTCAGCGGAGCGCAGTACATTTC3'
C69	...P L K P A K C...	IGF(C69)AS-43	5'CCCAAGCTTAAGCGCATTTAGCCGGTTTCAGCGGAGCGCAGTA3'
5' oligo (for the above muteins)		IGF(85p)28	5'GGGCATATGGGTCCGGAGACTCTGTGCG3'

(overlaps with IGF clone 85p-11 are underlined)

(SEQ ID NOS. 23 through 28 respectively)

Polymerase chain reaction (PCR) was performed in 100ul reactions containing 20 mM Tris pH 8.8, 10 mM KCl, 6 mM (NH4)2SO4, 1.5 mM MgCl2, 0.1% Triton X-100 using 20 pmole of each oligo, approximately 1ng of template DNA, 200uM each of dATP, dCTP, dGTP, TTP, 20pmole of each oligo primer, and 1ul (2.5U) of Pfu polymerase (Stratagene, San Diego, CA). The reactions were cycled 30 times for 1 min. at 95°C, 1 min. at 65°C and 1 min. at 72°C in a GeneAmp PCR System 9600 (Perkin Elmer Cetus). After the last cycle the reactions were held at 72°C for 10 min.

After PCR, the reaction mixtures were purified by passing through ChromaSpin 100 columns (Clontech Lab. Inc., Palo Alto, CA). Purified PCR fragments were digested with NdeI and HindIII and the ~210bp bands eluted from 1.5% agarose gel using NA45 paper (Schleicher and Schuell, Keene, NH) according to the manufacturer's specifications. The double cut and gel-purified DNA fragments were ligated to similarly cut and gel purified pT5T plasmid at 15°C for 18 hrs. The resulting ligation mixtures were used to transform E. Coli strain DH5a and plated on LB + ampicillin (50ug/ml) plates. Plasmid DNAs were prepared from several colonies using the Qiagen plasmid kit and the entire IGF-I gene sequenced with the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems). A correct construct was selected for each mutein (clones C11-1, C12-3, C15-1, C16-4, C55-1, C64-1, C65-1, C67-2, C69-1) and transformed into E. Coli strain BL21/DE3 for expression.

Preliminary expression studies of the muteins were performed by growing two representative transformants for each mutein in LB + tetracycline (12ug/ml) to an approximate OD_{600} 0f about 0.6-0.8. IPTG was added to a final concentration of 1mM and the cells allowed to grow for an additional 2 hrs. The IPTG induces the expression of T7 polymerase and the subsequent transcription and translation of the IGF muteins. Approximately 0.1 OD unit of the cells (both uninduced and IPTG-induced) were lysed in SDS sample buffer containing B-mercaptoethanol and electrophoresed on 15% SDS-PAGE. The gel was stained by Coomassie blue and the IPTG-inducible IGF mutein bands of the expected size were observed in lanes loaded with induced cells for each mutein.

EXAMPLE 3

Expression, Refolding, and Purification of Muteins

Although the following is written for the C3 mutein, the same procedure applies to other muteins contemplated by the instant invention. The only difference is in the starting cells used.

E. coli cells expressing the IGF-1 C3 mutein were suspended in Buffer A (50 mM Tris, pH 7.5, 20 mM NaCl and 1 mM dithiothreitol (DTT) at a concentration of 40 ml Buffer A to 10 g cell paste, and disrupted at 1800 psi using a French pressure cell (SLM Instruments, Inc., Urbana IL). The suspension was centrifuged at 20,000 X g for 30 min, and aliquots of the pellet and supernatant analyzed by SDS-PAGE. A major band corresponding to the IGF-1 C3 mutein was present in the pellet, but not the supernatant. The pellet was suspended in Buffer A at a concentration of 40 ml Buffer A to 10 g cell paste, and re-centrifuged at 20,000 X g for 30 min. This wash procedure was repeated 2 times. The final pellet containing the IGF-1 C3 mutein was suspended in 6 M guanidine, 50 mM Tris, pH 7.5, 6 mM DTT at a concentration of 25 ml to 10 g cells using a ground glass homogenizer. The suspension was incubated at room temperature for 15 min. The undissolved protein was removed by centrifugation at 20,000 X g for 30 min. The final concentration of the C3 mutein was 1.0 mg/ml. SDS-PAGE analysis of the pellet and supernatant following the procedure of Example 2 showed that IGF-1 C3 mutein was present in the supernatant only.

The denatured and reduced IGF-1 mutein was subjected to the following three-step refolding procedure:

1) Oxidized glutathione, the mixed-disulfide producing reagent (GSSG), was added to the supernatant to a final concentration of 25 mM, and incubated at room temperature for 15 min.

2) The solution was then diluted 10 fold gradually with 50 mM Tris, pH 9.7, and phenylmethylsulfonylfluoride and cysteine were added to final concentrations of 1mM and 5 mM, respectively. Final concentration of protein was 100ug/ml.

3) The refolding mixture was incubated overnight at 4oC, and then centrifuged at 20,000 x g for 15 min. SDS-PAGE analysis of the pellet and supernatant

showed that the supernatant was composed of relatively homogeneous IGF-1 C3 mutein.

Aliquots (50ul) of the supernatant were diluted to 200ul with Buffer B (0.05% TFA), injected onto a reverse phase column (RP-4, 1 x 250mm, Synchrom, Lafayette, IN), and eluted with 80% acetonitrile, 0.042% TFA (Buffer C) using a linear gradient (increase of 2% Buffer C/min) at a flow rate of 0.25 ml/min.

A single major peak representing refolded IGF-1 C3 mutein eluted at 26.5 min. Refolded IGF-1 C2 mutein eluted at 26.0 min. The retention times of refolded IGF-1 C3 and IGF-1 C2 muteins shifted to 32.2 min and 31.7 min, respectively, after being completely reduced and denatured in 5 M guanidine, 50 mM Tris pH 7.5, 100 mM DTT. These results indicate that both the C3 and C2 muteins refold to a single major species under the conditions described. N-terminal sequence analysis of IGF-1 C3 mutein eluting at 26.6 min gave the sequence: M G P C T L C (SEQ ID NO. 29) confirming that a cysteine residue has been substituted for glutamic acid in the 3 position of the N-terminal sequence of natural human IGF-1. An extra methionine residue is present at the N-terminus of the recombinant protein expressed by E coli. N-terminal sequence analysis of IGF-1 C2 mutein eluting at 26.0 min gave the sequence: G C E T L C (SEQ ID NO. 30) confirming that a cysteine residue has been substituted for proline in the 2 position of the N-terminal sequence of natural human IGF-1.

The refold supernatants containing the C55, C64, C65, C67 or C69 muteins were analyzed by diluting 50 ul aliquots of the supernatant to 200ul with Buffer B (0.05% TFA), injecting the diluted supernatant onto a reverse phase column (RP-4, 1 x 250mm, Synchrom, Lafayette, IN), and eluting with 100% acetonitrile, 0.042% TFA (Buffer D) using a linear gradient (increase of 2% Buffer D/min) at a flow rate of 0.25 ml/min. Two major distinct symmetrical peaks (1:1 ratio of each), PkI & PkII, eluted at 20.5 and 21.5 min, respectively. This pattern closely resembles the pattern observed for wild type ("WT") IGF-I (See, Meng, et al., J. Chromatography, Vol. 443, pp. 183-192 (1988)), specifically incorporated herein by reference. The earlier eluting peak observed in the WT refold has been shown to be an isomeric form of IGF-I with S-S assignments C6-C47, C48-C52, and C18-C61; whereas the later eluting peak is correctly refolded with S-S assignments C6-C48, C47-C52, C18-C61 (See, Raschdorf et al., Biomedical &

Environmental Mass Spectroscopy, Vol. 16, pp.3-8 (1988), specifically incorporated herein by reference).

An asymmetrical peak of varying size eluting at 21.5 - 23.0 min is also present in the refold supernatant. SDS-PAGE analysis of this material shows that it contains misfolded monomer and multimer forms of IGF-I. RP4 analysis of the refold supernatants containing the C11, C12, C15 or C16 muteins showed the presence of several peaks eluting from 20.5 - 21.5 min, as well as significant (50 to 75% of the total) amounts of apparently mis-folded material eluting at 21.5 - 23.0 min. The retention time of the refolded muteins shifted to 27 min after being completely reduced and denatured in 5 M guanidine, 50 mM Tris pH 7.5, 100 mM DTT. This demonstrates that the refolded forms "collapse" to a single polypeptide after reduction of disulfides. The RP4 peaks, therefore, represent distinct forms of IGF muteins with different disulfide bonds.

EXAMPLE 4

Isolation of Refolded IGF-1 Mutein

Refold mixtures (435 ml) prepared from 20g of E. coli paste containing either the refolded C3 or C2 mutein of Example 3 were concentrated to 100ml, acidified to pH 5.5 with 5M HCl, dialyzed against 20 mM sodium acetate, pH 5.5, and loaded onto an S-Sepharose (Pharmacia/LKB, Piscataway, NJ) column (1.6 X 15 cm) previously equilibrated with the sodium acetate buffer described above. The bound protein was eluted with a 300 ml linear gradient from 0 to 0.5M NaCl. Three ml fractions were collected. A single major protein peak eluted at 0.2-0.3M NaCl. Aliquots (100 ul and 25 ul) of each peak were analyzed separately by reverse phase (RP-4 1 X 250 mm) and gel filtration chromatography (Superdex 75 3.2 X 300 mm, Pharmacia/LKB, Piscataway, NJ). Gel filtration effectively separates monomers from dimeric and multimeric forms of IGF present in the refold supernatants. Fractions containing predominantly monomeric (determined from gel filtration and RP-4 analysis) C3 mutein were pooled, concentrated to 2.0 mg protein/ml, and 2.0-5.0 ml aliquots were loaded onto a Sephacryl S-100 (Pharmacia/LKB, Piscataway, NJ) gel filtration column (2.6 X 100 cm) previously equilibrated with 20 mM sodium acetate, pH 5.5, 250 mM NaCl. The protein was eluted at 2.0 ml/min, and aliquots (10ul) of each fraction were analyzed by RP-4 reverse phase chromatography and SDS-PAGE following the procedure of Example 2. Fractions containing a single refolded

species of IGF-1 C3 or IGF-1 C2 mutein monomer of 95% or more purity were pooled and concentrated to 250 ug/ml. This material was assayed for bioactivity and reacted with an 8.5 kDa polyethylene glycol as described below.

For the C11, C12, C15, C16, C55, C64, C65, C67 and C69 muteins, the refold supernatant was dialyzed into 20mM Tris, pH 7.4, concentrated 10-15X and loaded onto a Superdex 75 gel filtration column previously equilibrated with the same buffer. The monomers were then pooled and loaded onto an RP-4 column (RP-4, 2.1 x 250mm, Synchrom), and eluted with 100% acetonitrile, 0.042% TFA (Buffer B) using a linear gradient (increase of 2% Bufer B/min) at a flow rate of 1.0 ml/min. The C12, C55, C64, C65, C67 & C69 muteins refolded into 2 distinct RP-4 monomer peaks (1:1 ratio of each, PkI & PkII) closely resembling the pattern observed for WT-IGF-I (See Meng at al., cited above). The C12 & C15 monomer fractions from Superdex 75 also contained significant amounts of apparently mis-folded material eluting after PII (21.5 - 23.0 min). The C11, C15 and C16 monomer fractions contained multiple (3-6) peaks when analyzed by RP-4. The monomer peaks were collected separately and assayed for bioactivity and subjected to mass analysis (see below).

Mass analysis of the monomer peaks was performed using an API III obtained from Sciex, Toronto, Canada. Mass analysis was performed on both the refolded (disulfide bonds intact) and reduced and denatured monomers. The following masses were obtained:

TABLE 4

	w/cysteine mixed S-S			w/glutathione mixed S-S	
Mutein	Observed Reduced Mass (amu)	Expected Refolded Mass (amu)	Observed Refolded Mass (amu)	Expected Refoleded Mass (amu)	Observed Refolded Mass (amu)
C11 p1 C11 p2	7785	7906	7903 7904	8090	8088 8090
C12 p1 C12 p2	7770	7885	7885 7885	8075	8073 8074
C15 p1 C15 p2 C15 p3	7756	7877	7875 7848 7875	8061	8060 8033 8060
C16 p1 C16 p2	7736	7857	7855 7856	8041	8042 8042
C55 p1 C55 p2	7728	7849	7847 7847	8033	8032 8032
C64 p1 C64 p2	7771	7892	7888 7888	8076	8075 8074
C65 p1 C65 p2	7757	7878	7874 7875	8062	8059 8062
C67 p1 C67 p2	7813	7934	7932 7931	8118	8117 8119
C69 p1 C69 p2	7803	7918	7915 7915	8102	8100 8101

These masses of the reduced muteins match, within experimental error, the expected masses for a polypeptide with the indicated cysteine mutation. The masses of the refolded monomers match the expected mass of polypeptides having 3 intramolecular disulfide bonds and a single mixed disulfide of either cysteine - glutathione (Cys-S-S-Glutathione) or cysteine - cysteine (Cys-S-S-Cys). The mixed disulfides form during refolding and remain intact because there is no other cysteine residue present in the molecule available to form an intramolecular disulfide.

A scale up purification for the C69 mutein was also performed. The refold mixture from 8 gm of washed inclusion bodies (WIBS) was concentrated ~10X to 400 ml, dialyzed against 20mM sodium acetate, pH 5.5. 200 ul aliquots was loaded onto an Sephacryl S-100 (Pharmacia/LKB, Piscataway, NJ) gel filtration column (10 X 80 cm) previously equilibrated with 20 mM sodium acetate, pH 5.5 and 250 mM NaCl. The fractions were eluted at 25 ml per minute, and aliquots (50 ul) of each fraction were analyzed by SDS/PAGE. Fractions containing monomers were pooled.

To separate the two isomeric forms of C69, 200 ml of the S-100 monomer pool was diluted with 800 ml of 1.1 M ammonium sulfate-20mM sodium acetate, pH 5.5 (Buffer A) and loaded onto an Octyl Sepharose (Pharmacia/LKB, Piscataway, NJ) column (2.5 X 20 cm) previously equilibrated with Buffer A. The bound protein was eluted with a 750 ml linear gradient from Buffer A to 50% Buffer B (50% ethanol-20 mM sodium acetate, pH 5.5). 12 ml fractions were collected. Two major protein peaks eluted at 25% and 32% of Buffer B. Aliquots (50 ul) of each peak were analyzed by reverse phase (RP-4, 1 X 250 mm). Fractions containing predominantly correctly refolded (determined from RP-4 analysis) C69, eluting at ~32-38% Buffer B were pooled. Reverse phase analysis showed the correctly refolded C69 pool was 95% or more homogeneous. This material was assayed for bioactivity (see below).

EXAMPLE 5

Pegylation of Muteins

The C3, C2 and C69 muteins were covalently joined to an 8.5 kDa polyethylene glycol (8.5 kDa PEG) or an 20 kDa polyethylene glycol (20 kDa PEG) having a maleimide activating group in a two step process:

1) The purified IGF-1 muteins were partially reduced with DTT in a 15 ml reaction mixture containing 2.3 mg (296 nmoles) IGF-1 mutein, 170 ug DTT (1110 nmoles) in 14 mM sodium acetate, 33 mM sodium phosphate, pH 7.0. The final concentration of protein was 10 ug/ml, and the molar ratio of DTT:protein was 3.75:1. For reaction with the C69 mutein, 91ug DTT (592 nmoles) was used and the molar ratio of DTT:protein was 2:1. The reaction mixture was incubated at room temperature for 3 hours (5 hours for reaction with the C69 mutein) and terminated by the addition of 1.0 ml of 1M sodium acetate, pH 5.5. The reaction mixture was dialyzed at 4°C overnight against 20 mM sodium acetate pH 5.5.

2) The partially reduced IGF-1 mutein was reacted with either the 8.5 kDa PEG or the 20 kDa PEG in a 20 ml reaction mixture containing 2.3 mg (296 nmoles) of protein, 9.985 mg (1174 nmoles) 8.5 kDa PEG in 15 mM sodium acetate, 26 mM sodium phosphate, pH 7.0. The final concentration of protein was 112 mg/l. The molar ratio of 8.5 kDa PEG:protein was 4:1; for reaction with the C69 mutein the molar ratio of 20kDa PEG:protein was 4:1. The reaction mixture was incubated at room temperature for 3 hours, and terminated by placing at 4oC or freezing at -20°C. SDS-PAGE analysis of the reaction mixture following the procedure of Example 2 showed that approximately 50% of the partially reduced PEGylated C2 and C3 mutein was converted to a mono-PEGylated species. The C3 and C2 20kDa-PEG conjugates migrated at a relative molecular weight of approximately 60kDa on SDS PAGE; The C3 and C2 8.5kDa-Peg conjugates migrated at a relative molecular weight of about 23 kDa on SDS PAGE. Approximately 20% of the partially reduced PEGylated C69 mutein was converted to a mono-PEGylated species migrating at a relative molecular weight of 67 kDa on SDS PAGE.

Wild type IGF-1 subjected to the same partial reduction conditions and PEGylation procedures did not become PEGylated.

EXAMPLE 6

Purification of Pegylated Muteins

The pegylated C2 or C3 mutein reaction mixtures (containing approximately 100-200 mg protein) were dialyzed extensively at 4°C against 5 mM citric acid, pH 2.6. The pegylated mutein was separated from the unPEGylated mutein using an S-Sepharose (Pharmacia/LKB, Piscataway, NJ) cation exchange column (2.5 X 25 cm) previously

equilibrated with 5 mM citric acid buffer, pH 2.6. The bound protein was eluted with a 2000 ml linear gradient from 0 to 1 M NaCl. 25 ml fractions were collected. Pegylated C2 or C3 muteins eluted at 0.25-0.4M NaCl and the unpegylated protein eluted at 0.8-0.9 M NaCl.

Fractions containing the pegylated C2 or C3 muteins were pooled, concentrated and further purified by Sephacryl S-200 gel filtration chromatography. 15 ml of the concentrated fractions containing approximately 20 mg of total protein was loaded onto a Sephacryl S200 (Pharmacia/LKB, Piscataway, NJ) column (2.6 X 100 cm) previously equilibrated with 20 mM sodium acetate, pH 5.5 containing 250 mM NaCl. The protein was eluted at 2.0 ml per min. The bulk of the material eluted with an apparent MW of 200 kDa.

The C69-PEG was separated from the unpegylated C69 mutein by Q-Sepharose anion exchange chromatography . 100 ml of the reaction mixture containing 11 mg of total protein was loaded onto a Q-Sepharose anion exchange column column (Pharmacia/LKB, Piscataway, NJ) column (2.6 X 100 cm) previously equilibrated with 20mM Tris, pH 9.0 (buffer A). The bound protein was eluted with a linear gradient of 20mM tris Ph 9.0, 1M NaCl (buffer B) at 5.0 ml/min. 10 ml fraction were collected. The C69-PEG eluted at 50mM NaCl, well separated from the unreacted monomer which eluted at 100mM NaCl. C69-PEG was pooled, concentrated to 13 ml and loaded onto an S-200 gel filtration column (2.6 x 100cm) previously equilibrated with 20mM sodium acetate, ph 5.5, 250 mM NaCl.

EXAMPLE 7

Bioassay of Pegylated Muteins

Recombinant human metIGF-1 (rIGF-1) (Bachem California, Torrance, CA), various unpegylated and PEGylated muteins were tested for their relative mitogenic activity and affinity for recombinant insulin-like growth factor binding protein 1 ("IGF-BP1"), which is described in PCT Application publication WO 89/09792, published on October 19, 1989.

A. Relative Mitogenic Activity

The relative mitogenic (growth stimulating) activities of the C3 and C2 muteins and pegylated C3 and C2 muteins were compared to that of wild type IGF-1 by measuring the relative amount of ^{3}H-thymidine incorporated into rat osteosarcoma cells when varying amounts of the proteins were present under serum free conditions. The rat osteosarcoma

cells (the UMR106 cell line; obtained from American Type Culture Collection, Accession No. CRL-1661, Rockville, Maryland) were plated at 5-6 X 104 cells in 0.5 ml of Ham's F12 Media (Mediatech, Herndon, VA) containing 7% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin and 2 mM L-glutamine per well in 48-well tissue culture plates (Costar, Cambridge, MA). After incubating for 72 hours at 37°C when the cells were confluent, the cells were washed twice with phosphate buffered saline (PBS) and pre-incubated in serum-free Ham's F12 medium containing 100 U/ml penicillin and 100 mg/ml streptomycin and 2 mM L-glutamine for 24 hours. After the pre-incubation, 0.5 ml of F12 serum-free medium containing serial dilutions (1.0 - 1,000 ng/ml) of rIGF-1, C3 and C2 muteins, and pegylated C3 and C2 muteins were incubated separately for an additional 20-24 hours. Each well was then pulse labeled with 0.5 uCi of 3H-thymidine (NEN Research Products, DuPont Co., Boston, MA) for 4 hours, then washed with cold PBS three times and incorporated 3H-thymidine was precipitated with cold 7% trichloroacetic acid (J.T. Baker Inc., Phillipsburg, NJ). After rinsing with 95% ethanol, cells were solubilized with 0.3 M NaOH and aliquots removed for scintillation counting. 3H-thymidine was quantitated by liquid scintillation counting. All assays were performed in triplicate.

The C3 and C2 muteins and pegylated C3 and C2 muteins were found to stimulate the same maximal level of 3H-thymidine incorporation into DNA as recombinant IGF-1. The potencies of the C3 and C2 muteins and the pegylated C3 and C2 muteins were about 3 to 4 fold lower than recombinant IGF-1. The ED50 (dose required for half maximal activity) of recombinant IGF-1 was 5-10 ng/ml compared with 30-40 ng/ml for unpegylated C3 and C2 muteins, and the pegylated C3 and C2 muteins.

These experiments demonstrate that the mitogenic activity of IGF-1 has been substantially retained by the C3 and C2 muteins and the pegylated C3 and C2 muteins. All four molecules are capable of simulating cells to divide, as measured by ^{3}H-thymidine incorporation into DNA. All four molecules are capable of stimulating cells to divide to the same maximal extent.

Using the assay described above, the relative mitogenic activity of of the RP-4 peaks (described above) of the C11, 12, C15, C16, C55, C64, C65, C67 and C69 IGF muteins and of C69-PEG was also determined. The results of the latter assay is set forth in Table 5.

TABLE 5

APPPROXIMATE ED_{50} OF IGF-I MUTEIN PEAKS & C69-PEG (NG/ML)

	PEAK I (20.5 min)	PEAK II (21.5 min)	PEAK III (22.0 min)	PEAK IV (22.5 min)
C11	~200	~200	~200	ND
C12	50 - 55	6 - 8	ND	ND
C15	~150	~70	~5	~300
C16	~20	~8	ND	ND
C55	~40	5 - 7	~40	ND
C64	~90	~22	ND	ND
C65	~45	~45	ND	ND
C67	~25	5 - 6	ND	ND
C69	20 - 25	5 - 6	ND	ND
C69-PEG		5-6		
rWT IGF-I	20	4-6		

WT IGF-I ED_{50} ~4-6 ng/ml

ND - NOT DETERMINED

The mitogenic activity of Peaks II of the C12, C16, C55, C67 and C69 mutein monomers was not significantly different from the mitogenic activity of correctly refolded WT rIGF-I (Peak II). The ED_{50} of wild type rIGF-I was 4-6 ng/ml compared with 5-8 ng/ml for these unPEGylated mutein monomers. Table 5 shows that Peaks I of the various muteins had lower (5-30 fold) bioactivity than correctly refolded WT rIGF-I similar to the bioactivity of WT rIGF-I peak I. The C69-PEG conjugate, synthesized from peak II of C69, had the same bioactivity as C69 peak II and correctly refolded WT rIGF-I.

B. Relative Affinity for IGF-BP1

The relative affinities of the C3, C2 and C69 muteins and the PEGylated forms of those muteins for IGF binding protein-1 (IGF-BP1) were compared to that of the wild type IGF-1 by measuring the ability of IGF-BP1 to inhibit the mitogenic activities of the proteins on rat osteosarcoma cells. The rat UMR106 osteosarcoma cells were plated at 5-6 X 104 cells in 0.5 ml of Ham's F12 containing 7% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin and 2 mM L-glutamine per well in 48-well tissue culture plates. After incubating for 72 hours at 37°C when the cells became confluent, the cells were washed twice with PBS and pre-incubated in serum-free Ham's F12 medium containing 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine for 24 hours. After the pre-incubation, 0.5 ml of F12 serum-free medium containing either 50 ng/ml or 200ng/ml of rIGF-1, C3 or C2 mutein, or pegylated C3 or C2 mutein were incubated separately with varying amounts of IGF-BP1 (100 ng/ml - 1 X 104 ng/ml) for an additional 20-24 hours. Each well was then pulse labeled with 0.5 uCi of 3H-thymidine (NEN Research Products, Du Pont Co., Boston, MA) for 4 hours, then washed with cold PBS three times and incorporated 3H-thymidine was precipitated with cold 7% trichloroacetic acid (J.T. Baker Inc., Phillipsburg, NJ). ^{3}H-thymidine was quantitated by liquid scintillation counting. All assays were performed in triplicate.

The results of this experiment indicated that the affinities of the unpegylated C3 mutein and the pegylated C3 mutein for IGFBP1 were greatly reduced. At a molar ratio of 20:1 (IGFBP1:rIGF-1), the mitogenic activity of rIGF-1 (50 ng/ml) was reduced 80%; however, the mitogenic activities of the same concentrations of the unpegylated C3 mutein and pegylated C3 mutein were reduced 35% and 0%, respectively. Similarly, when 200 ng/ml of the proteins were incubated with a 20 fold molar excess of IGF-BP1, 70% of the

mitogenic activity of rIGF-1 was inhibited, whereas none of the mitogenic activity of the pegylated C3 mutein was inhibited. The affinities of of both the unpegylated C2 mutein and the pegylated C2 mutein were identical to that of wild type IGF-1. The affinity of both the unpegylated C69 and C69-PEG for IGF-BP1 were not significantly different from that of WT rIGF-I

These data indicate that the pegylated C3 mutein has greatly reduced affinity for IGFBP1 when compared to IGF-1. Thus the mitogenic activity of the pegylated C3 mutein will not be inhibited by IGF binding proteins under conditions where the mitogenic activity of IGF-1 will be inhibited. However, the affinity of pegylated C2 and C69 muteins for IGFBP1 is the same as the affinity of wild type IGF-1. Thus the mitogenic activity of pegylated C2 and C69 muteins will be inhibited by IGF binding proteins under the same conditions where the mitogenic activity of IGF-1 will be inhibited.

EXAMPLE 8

Animal Tests

Animal studies were performed to compare the pharmacodynamic properties of the muteins and PEG conjugates of the present invention to the pharmacodynamic properties of wild type IGF-1.

A. Animals

Male Sprague Dawley rats with pituitary glands surgically removed (hypophysectomized or Hypox rats) and weighing 110-121 grams were obtained from Charles River Co. The rats were maintained in cages with lighting controlled over a 12 h-light/12 h-dark cycle.

The animals had continuous access to water and food. Five animals were housed per cage. The weights of the rats were monitored daily and only rats with weight gains of less than 2 grams per week during the 2-3 weeks after arrival were considered to be succesfully hypophysectomized and used for the experiments.

B. Methods

In Experiment I, animals (10 Hypox rats per group) were injected every third day (ETD) subcutaneously (sc) with WT rIGF-I (160 mg, 320mg), unpegylated C2 (320mg),

unpegylated C3 (320mg), pegylated C2 0(C2-PEG, 320mg) or Pegylated C3 (C3-PEG,320mg) dissolved in 0.2 ml of binding buffer (0.1 M HEPES-0.05 M NaH_2PO_4). A separate group of 10 animals received 0.2ml vehicle. Injections were given between 0700 hours and 0800 hours and body weights were recorded daily between 1600 h and 1700 h. The weights of rats on the day after the last injection were taken as the final weight.

In Experiment II, animals (9 Hypox rats per group) were injected every third day subcutaneously with WT rIGF-I (320mg, single injection daily, SID; 320mg ETD; 640mg ETD), or C3-PEG (320mg ETD, 640mg ETD, 960mg ETD).dissolved in 0.2 ml of binding buffer (0.1 M HEPES-0.05 M NaH_2PO_4). A separate group of 9 animals received 0.2ml vehicle. Injections were given between 0700 h and 0800 h and body weight were recorded daily between 1600 h and 1700 h. The weights of rats on the day after the last injection were taken as the final weight.

In Experiment III, animals (10 Hypox rats) were injected every third day subcutaneously with C3-PEG (160mg ETD, 320mg ETD), dissolved in 0.2 ml vehicle (0.1 M HEPES-0.05 M NaH_2PO_4). A separate group of 10 animals received 0.2ml vehicle. Injections were given between 0700 h and 0800 h and body weight were recorded daily between 1600-1700 h. The weights of rats on the day after the last injection were taken as the final weight.

At the end of Experiments I & II, rats were asphyxiated with CO_2 and weighed. In Experiment III, the tibia were removed and the epiphyseal width measured.

C. Results

Experiment I: Rats treated with sc injections with either 160mg or 320mg of WT IGF-I ETD showed no significant weight gain compared with animals injected with vehicle (Table 6). Similarly, animals injected ETD with 320 mg un-PEGylated C2 IGF-I or un-PEGylated C3 mutein did not show significant weight gain. Animals injected ETD with 320 mg C2-PEG and C3-PEG gained 4.42 $\pm$ 0.74 g and 5.45 $\pm$ 0.98 g, respectively, which was significantly greater than the weight gain of animals injected with wild type IGF-1 ($p<0.01$). The weight gain of animals injected with PEGylated C2 or PEGylated C3 was significantly greater than the weight gain of animals injected with the un-PEGylated C2 or C3 mutein ($p<0.05$). The PEGylated proteins clearly showed efficacy; however, the

identical dose of WT IGF-I showed no efficacy. Surprisingly, the addition of PEG improves the biological potency of the molecule.

TABLE 6

THE EFFECT OF IGF-I MUTEINS (UNPEGYLATED & PEGYLATED) ON THE GROWTH OF HYPOPHESECTOMIZED RATS

MOLECULE	DOSE ug/day	FREQUENCY	MEAN WT GAIN (g)	P VALUE vs
Vehicle		ETD	-1.28 ± 0.95	
WT IGF-I	160	ETD	0.23 ± 0.87	
WT IGF-I	320	ETD	0.59 ± 0.79	
C2	320	ETD	-0.52 ± 0.67	
C2-PEG	320	ETD	4.42 ± 0.74	WT 320 0.01 C2 320 0.01 C3 320 <0.05
C3	320	ETD	1.75 ± 0.90	
C3-PEG	320	ETD	5.45 ± 0.98	WT 320 <0.01 C2 320 <0.05 C3 320 < .05

These results demonstrate that the PEGylated muteins exhibit enhanced pharmacodynamics over WT IGF-I and the un-PEGylated IGF muteins.

Experiment II: Rats treated with sc injections of WT IGF-I 320mg SID, 320mg ETD and 640mg ETD gained 4.02g ± 0.46g , 0.81g ± 0.81g and 1.41g ± 0.52g, respectively (Table 7). However, animals given 160mg, 320mg, 640mg 960mg of C3-PEG ETD gained 5.22g + 0.46g, 5.50g ± 0.52g, 8.69g ± 0.67g, and 10.43g ± 0.77g, respectively (Table 6). All doses of C3-PEG ETD stimulated significantly more weight gain than both WT

IGF-I doses given ETD. Animals injected with either 640mg or 960mg of C3-PEG ETD gained significantly more weight than those given 320mg WT IGF-I SID. C3-PEG doses of 160mg and 320mg ETD stimulated greater weight gain than 320mg of WT IGF-I SID; however, these differences did not reach statistical significance.

TABLE 7

THE EFFECT OF C3-PEGylated IGF-I ON THE GROWTH OF HYPOPHESECTOMIZED RATS

MOLECULE	DOSE ug/day	FREQUENCY	MEAN WT GAIN (g)	P VALUE vs
Vehicle		ETD	-0.22 ± 0.38	
WT IGF-I	320	SID	4.02 ± 0.46	WT 320 ETD 0.01 WT 640 ETD 0.01
WT IGF-I	320	ETD	0.81 ± 0.81	
WT IGF-I	640	ETD	1.41 ± 0.52	
C3-PEG	160	ETD	5.22 ± 0.46	WT 320 ETD 0.01 WT 640 ETD 0.01
C3-PEG	320	ETD	5.50 ± 0.52	WT 320 ETD 0.01 WT 640 ETD 0.01
C3 PEG	640	ETD	8.69 ± 0.67	WT 320 SID <0.01 C3PEG 160 ETD <0.01 C3PEG 320 ETD <0.01
C3-PEG	960	ETD	10.43 ± 0.77	WT 320 SID <0.01 C3PEG 160 ETD <0.01 C3PEG 320 ETD <0.01

Experiment II demonstrates that C3-PEG administered sc ETD exhibits greater potency than WT IGF-I administered sc ETD. All doses of C3-PEG stimulated greater mean weight gain than animals given 320mg WT IGF-I SID. The enhanced pharmacodynamics of C3-PEG make it more potent than WT IGF-I in the animal model described.

Experiment III: Rats treated with sc injections of C3-PEG 160 mg and 320 mg gained 8.3g ± 0.7g and 9.0g ± 0.6g, respectively (Table 4). Vehicle gained 4.2g + 0.3g. The weight gained induced by C3-PEG was statistically greater than animals given vehicle. Similarly, the tibial epiphyseal widths of rats receiving C3-PEG were statistically greater than rats receiving vehicle (Table 8).

TABLE 8

THE EFFECT OF C3-PEGylated IGF-I ON THE GROWTH OF HYPOPHESECTOMIZED RATS (WEIGHT GAIN)

MOLECULE	DOSE ug/day	FREQUENCY	MEAN WT GAIN (g)	MEAN TIBIA WIDTH (mm)	P VALUE vs
Vehicle		ETD	4.2 ± 0.3	0.136 ± 0.004	
C3-PEG	160	ETD	8.3 ± 0.46	0.159 ± 0.008	Vehicle <0.01
C3-PEG	320	ETD	9.0 ± 0.g	0.151 _ 0.004	Vehicle <0.01

TABLE 9

THE EFFECT OF C3-PEGylated IGF-I ON THE GROWTH OF HYPOPHESECTOMIZED RATS (TIBIA EPIPHYSEAL WIDTH)

MOLECULE	DOSE ug/day	FREQUENCY	MEAN TIBIAL WIDTH (mm)	P VALUE vs
Vehicle		ETD	0.136 $\pm$ 0.008	
C3-PEG	160	ETD	0.180 $\pm$ 0.01	Vehicle <0.01
C3-PEG	320	ETD	0.167 $\pm$ 0.06	Vehicle <0.01

Experiment III demonstrates that C3-PEG stimulates not only weight gain, but also bone growth in HYPOX rats. This indicates that C3-PEG may be a useful pharmaceutical for the induction of bone formation.

Although this invention has been described with respect to specific embodiments, it is not intended to be limited thereto. Various modifications which will be apparent to those skilled in the art are deemed to fall within the spirit and scope of the present invention.

Claims:

1. A polyethylene glycol (PEG) conjugate comprising PEG and a mutein of IGF, said PEG is attached to said mutein at a free cysteine.

2. The conjugate of claim 1, wherein said PEG is attached to the free cysteine through an activating group selected from the group consisting of maleimide, sulfhydryl, thiol, triflate, tresylate, aziridine, exirane, and 5-pyridyl.

3. The conjugate of claim 1, wherein the IGF is IGF-1.

4. The conjugate of claim 1, wherein the PEG has a molecular weight selected from the group consisting of 5 kDa, 8.5 kDa, 10 kDa, and 20 kDa.

5. The conjugate of claim 4, wherein the PEG has a molecular weight of 8.5 kDa.

6. The conjugate of claim 1, further comprising a second polypeptide attached to said PEG.

7. The conjugate of claim 6, wherein the second polypeptide is a mutein of IGF.

8. A mutein of IGF having a non-native cysteine.

9. The mutein of claims 8, wherein said non-native cysteine is in the N-terminal region of the mutein.

10. The mutein of claim 8, wherein said mutein is a recombinant product.

11. The mutein of claim 8, wherein said mutein is expressed by E. coli.

12. The mutein of claim 8, wherein said non-native cysteine is in the C-terminal region of the mutein.

13. The mutein of claim 12, wherein said mutein is the C69 mutein.

14. A method of making the conjugate of claim 1, comprising attaching PEG to a free cysteine of an IGF mutein, said mutein having a non-native cysteine in the N-terminal region.

15. The method of claim 14, wherein said PEG is attached to the free cysteine through an activating group selected from the group consisting of maleimide, sulfhydryl, thiol, triflate, tresylate, aziridine, exirane, and 5-pyridyl.

16. The method of claim 14, wherein the activating group is maleimide.

17. The method of claim 14, wherein the PEG is attached to an IGF mutein and another polypeptide.

18. The method of claim 17, wherein the other polypeptide is an IGF mutein.

20. A pharmaceutical composition comprising the conjugate of claim 1 in a pharmaceutically acceptable carrier.

21. A method for treating an IGF associated condition comprising administering the pharmaceutical composition of claim 20 to a patient.

INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/US 95/06540

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO-A-94 12219 (SYNERGEN INC ;COX GEORGE N (US); MCDERMOTT MARTIN J (US)) 9 June 1994 see page 7 see page 8, line 20 - page 9, line 2 see page 9, line 16 - page 10, line 9; claims 1,2,11,18,19,21,22,24 ---	1-21
A	WO-A-90 12874 (GENETICS INST) 1 November 1990 see page 7, line 19 - line 28 see page 13, line 20 - line 33 --- -/--	1,2,15, 16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 September 1995	26. 09. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Berte, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern. al Application No
PCT/US 95/06540

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO-A-94 12219 (SYNERGEN INC ;COX GEORGE N (US); MCDERMOTT MARTIN J (US)) 9 June 1994 see page 7 see page 8, line 20 - page 9, line 2 see page 9, line 16 - page 10, line 9; claims 1,2,11,18,19,21,22,24	1-21
A	WO-A-90 12874 (GENETICS INST) 1 November 1990 see page 7, line 19 - line 28 see page 13, line 20 - line 33	1,2,15, 16

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 September 1995	26. 09. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Berte, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/US 95/06540

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO-A-94 22466 (SYNERGEN INC ;COX GEORGE N (US); RUSSELL DEBORAH A (US)) 13 October 1994 see page 8, line 12 - line 22 see page 9, line 1 - line 6 see page 13, line 8 - line 14; claims 1,5-13,17-20,25,28,29,31	1-21
A	J. BIOL. CHEM. (1994), 269(10), 7610-16 CODEN: JBCHA3;ISSN: 0021-9258, 1994 KUAN, CHIEN TSUN ET AL 'Pseudomonas exotoxin A mutants. Replacement of surface exposed residues in domain II with cysteine residues that can be modified with polyethylene glycol in a site-specific manner' see page 7611	1-21
A	BIO/TECHNOLOGY (1990), 8(4), 343-6 CODEN: BTCHDA;ISSN: 0733-222X, 1990 GOODSON, ROBERT J. ET AL 'Site-directed PEGylation of recombinant interleukin-2 at its glycosylation site' cited in the application	1,2,8, 10,11,14
P,A	TETRAHEDRON LETT. (1994), 35(35), 6515-16 CODEN: TELEAY;ISSN: 0040-4039, 1994 TANG, WEI ET AL 'Preparation of a new PEGylation reagent for sulfhydryl-containing polypeptide' see the whole document	1-21
A	WO-A-92 16221 (SYNERGEN INC) 1 October 1992 see page 19, line 5 - line 19 see page 21, line 6 - line 15 see page 28, line 6 - page 29, line 26 see page 30, line 12 - line 27 see page 33, line 14 - line 37	1-21

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/US 95/06540

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9412219	09-06-94	AU-B-	6048294	22-06-94
		CA-A-	2149048	09-06-94
WO-A-9012874	01-11-90	US-A-	5166322	24-11-92
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		EP-A-	0668354	23-08-95
		JP-T-	4504801	27-08-92
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WO-A-9216221	01-10-92	AU-A-	1674292	21-10-92
		EP-A-	0575545	29-12-93
		JP-T-	6506218	14-07-94
		NO-A-	933270	01-11-93

INTERNATIONAL SEARCH REPORT

Int .ational application No.
PCT/US 95/ 06540

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
REMARK: Although claim 21 is directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.